VIROLOGY 222, 289–292 (1996)
ARTICLE NO. 0423

SHORT COMMUNICATION

Fine Mapping of a C-Terminal Linear Epitope Highly Conserved among the Major Envelope Glycoprotein E2 (gp51 to gp54) of Different Pestiviruses

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Received March 22, 1996; accepted May 22, 1996

Envelope glycoprotein E2 (gp51 to gp54) is the major neutralizing antigen of pestiviruses, which include classical swine fever virus (CSFV), bovine viral diarrhoea virus (BVDV), and border disease virus (BVD). Previous studies carried out using a panel of monoclonal antibodies raised against CSFV strain Brescia have revealed the existence of four antigenic domains, A to D, of the E2 protein, all of which are located at the N-terminal half of the molecule. Here we report the detailed mapping, using three complementary techniques, of a novel linear epitope located at the C-terminal part of the molecule, which reacted with a monoclonal antibody (4-9D4) as well as polyclonal animal sera. This epitope is highly conserved in the three different members of pestiviruses and hence can be used as a genus-specific diagnosis tool. The observation that this epitope is not accessible on the native virus surface, together with its C-terminal location, supports a recently proposed structural model, indicating that the C-terminal part of E2 is membrane-bound while the N-terminal half of the molecule is exposed on the virus surface.

The genus *Pestivirus* of the *Flaviridae* family is composed of three members, i.e., classical swine fever virus (CSFV), bovine viral diarrhoea virus (BVDV), and border disease virus (BDV) (*1*). Pestiviruses are structurally and antigenically closely related. Antibodies derived from animals infected with one group of viruses often cross-react with the other members of the genus (*2*). However, most monoclonal antibodies (mAb) directed against structural proteins are able to discriminate between CSFV and BVDV (*3–5*).

The genome of pestiviruses is a positive-stranded RNA of about 12.5 kb (*6–8*). The genome codes for a single open reading frame (ORF) flanked by two short noncoding regions. The open reading frame is translated into a polyprotein of approximately 4000 amino acids in length, which is then processed into individual polypeptides by virus- and cell-encoded proteases (*9*). It has been suggested that the nomenclature for pestivirus glycoproteins to change from molecular mass-based names (e.g., gp53) to position-based names (e.g., E2) (*9*). The coding regions for the three envelope glycoproteins E0 (gp44–48), E1 (gp31–33), and E2 (gp51–54) have been mapped for CSFV (*8–10*). The major envelope glycoprotein E2 is located between amino acid residues 690 and 1060 of the single ORF polyprotein (*7, 11*). Among the three envelope glycoproteins, E2 is the most antigenic, and an immune response against E2 alone is sufficient for protection (*12–14*). Therefore, E2 has long been considered an ideal candidate for recombinant vaccine development as well as for serum diagnosis.

Using mAbs directed against the E2 protein of CSFV strain Brescia, four antigenic domains, A to D, have been identified on the E2 protein (*15–18*). All of them are located in the N-terminal half of the molecule between amino acid residues 690 and 866. The N-terminal half of the molecule has been shown to be important for virus neutralization (*15, 18*). From their epitope mapping data, Van Rijn *et al.* (*18*) have recently proposed a structural model for the E2 protein, which provides the location of the four antigenic domains, the potential glycosylation sites, and the important disulfide bonds. In this model, the C-terminal 35 amino acid residues are buried in the membrane envelope.

In this paper we report the identification and detailed mapping of a linear epitope located at the C-terminal part of the E2 protein. This represents a new antigenic region of the E2 protein which has not been reported before. Our epitope mapping results are consistent and complementary to the previous data which led to the proposal of the above mentioned structural model of E2.

The mAb 4-9D4 used in this study was raised in mice against a denatured recombinant E2 protein produced in yeast using an cDNA clone coding for amino acid residues 544–1093 of E2 protein from CSFV strain Weybridge (*19;* also M. Yu and J. White, unpublished results). Initial characterization of the mAb indicated that it recognized a linear epitope of the E2 protein, which is not accessible in the native virus. In Western blot, mAb 4-

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0042-6822/96 $18.00

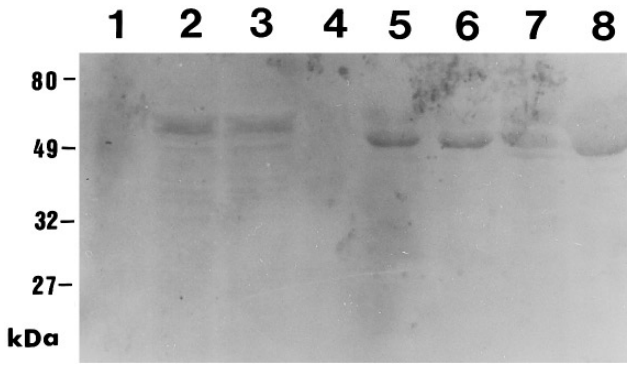

FIG. 1. Western blot analysis of pestivirus E2 proteins using mAb 4-9D4. Preparation of viral antigens were carried out using standard methods. Cell pellets, both infected or noninfected, were treated with NP-40 to release viruses. Western blot was carried out as described in (*20*). Lane 1, PK15 cell lysate, used as a negative control; Lanes 2 and 3, CSFV strains NSW and Baker, respectively; Lane 4, Lamb testis (LT) cell lysate, used as negative control; Lanes 5–7, BVDV strains NADL, Singer, and Osloss, respectively; Lane 8, BDV strain BD78. The numbers given at the left are molecular weight standards determined from the prestained molecular weight markers obtained from Bio-Rad.

9D4 not only reacted with the recombinant E2 protein originally used for immunization (data not shown), but also reacted with viral E2 proteins derived from all pestivirus strains available for testing in our laboratory (see Fig. 1). The mAb failed to react in ELISA with purified native virus particles (C. Morrissy, unpublished results). However, positive ELISA results could be obtained after the purified virions were first treated with 1% NP-40 for 2 hr at room temperature. When cell monolayer was treated with 2–3% formalin and 0.1% NP-40, positive results were also obtained in immunoperoxidase test (C. Morrisy, unpublished results). These results suggest that the 4-9D4 epitope is not accessible on the surface of the native virions.

To map the epitope defined by mAb 4-9D4, three complementary mapping techniques were employed. These include the use of two different types of phage display epitope libraries followed by the application of overlapping synthetic peptides in competition ELISA analysis.

First, a gene-targeted phage display epitope library was constructed using the procedures previously developed by our group (*20, 21*). Briefly, using the 1.7-kb cDNA of CSFV E2 cloned in pUC vector (*19*) as starting material, DNase I partial digestion was conducted to enrich for random fragments in the range of 100–200 bp. After end-repairing, the blunt-ended small fragments were inserted into the phage display vector fUSE1 (*22*), which was cut with *Pvu*II and dephosphorylated with calf intestine alkaline phosphatase, to obtain a random epitope library containing approximately 5×10^5 independent phage clones. After two rounds of affinity selection using mAb 4-9D4, 8 positive clones were selected which displayed peptides reactive with 4-9D4 in both ELISA and Western blot (data not shown). DNA sequencing analysis indicated that 7 of the 8 clones contained an identical insert coding for a 21-aa peptide (KNKYYEPRDSYFQQYMLKGEY) which corresponds to amino acid residues 991–1012 of the CSFV E2 protein (*7, 19*). The 8th clone contained a larger insert corresponding to amino acid residues 985–1051, which covered the smaller 21-aa insert sequence isolated in the other 7 clones. Further analysis was, therefore, concentrated on the clone containing the 21-aa insert, which was named f1CSFV-D4. Recombinant phage particles were purified from f1CSFV-D4 by three times PEG precipitation and tested for their reactivity with polyclonal animal sera. Positive results were obtained, in ELISA and Western blot, with polyclonal sera raised against CSFV and/or BVDV in pig and goat (data not shown).

Second, mAb 4-9D4 was also used to select for mAb-specific peptides from a synthetic peptide phage display library obtained from G. Smith (S. Choukri and G. Smith, unpublished results). This library was constructed by inserting random oligonucleotide fragments, coding for random 15-mer peptides, into the gene VIII display vector f88-4 (*23*). After two rounds of affinity selection as described (*20, 22*), positive clones were picked up by a colony blotting procedure (*24*), followed by confirmation with ELISA analysis. Twelve positive clones were then selected for further analysis by sequencing. As shown in Fig. 2A, these clones represent 10 different classes of inserts, all of them contained a 4-amino acid residue core consensus region, highly homologous to the YYEP sequence in the E2 protein of CSFV. These results not only corroborated the data obtained above from the E2-targeted phage display epitope library, but also revealed that YYEP is the critical binding site for 4-9D4. It is interesting to note that there was not much sequence homology in the flanking regions of the 15-mer inserts, suggesting that the YYEP tetra-peptide is sufficient for mAb 4-9D4 recognition. The results also indicated that among the YYEP tera-peptide sequence, the second position (occupied by Y) allows limited sequence variation (see more discussion below).

Sequence alignment, in Fig. 2B, for the YYEP-region from E2 proteins of all pestiviruses whose E2 gene has been sequenced indicated a strong sequence conservation in this region. This provides molecular evidence for our initial finding that mAb 4-9D4 was able to react with E2 proteins from all the pestiviruses available for testing in our laboratory (Fig. 1). Furthermore, the only sequence variation in this region, i.e., the replacement of the Y residue in CSFV strains, at the second position of the YYEP tetra-peptide, by the F residue in some BVDV strains is in support of the finding obtained from the studies of random phage-displayed peptides as shown in Fig. 2A.

In parallel, a third approach, utilizing a multipin peptide synthesis system (*25*), was also used. Briefly, overlapping 8-mer peptides, offset by one, spanning residues 989–1012 of CSFV E2 protein were synthesized on polyethylene pins with a linker allowing cleavage of the peptides

A

Clone	Sequence
f1CSFV-D4	KNK YYEP RDSYFQQYMLKGEY
f88-D4-1	TDATTIMPPPQG ....
f88-D4-2	RP .A.. WGWTLAHDA
f88-D4-3	.W.. H.VMWTDPVHA
f88-D4-4/12	S.N .... ELYTPSNL
f88-D4-5	ESISPQS .... IGRS
f88-D4-6/9	MQQ .... S.MMVDLE
f88-D4-7	EPSSTL .... GR.VQ
f88-D4-8	GP .A.. G.GH.SLQY
f88-D4-10	SANGF .... GATMYK
f88-D4-11	.... YETRTIIPSWH

B

Virus	Strain	Sequence
CSFV	Weybridge	KNK YYEP RDSYFQQYMLKGEY
	Brescia	R.R ....
	C	..R ....
	GPE	... .
	LPC	..R ....DI.MA..
	ALD	... .
	Alfort	... .
BVDV	Singer	... .F.. .....H.....
	SD-1	... .F.. .....D.
	Osloss	... .N.....
	NADL	... .F.. .....
	C24V	... .F.. .....
BDV	BD78	P.. .... ..R.S.....W

FIG. 2. Sequence analysis of the 4-9D4 epitope. (A) Alignment of peptide sequences isolated from synthetic peptide phage display library. The top sequence (f1CSFV-D4) is the epitope fragment isolated from the E2-targeted phage display library, which corresponds to amino acid residues 991–1012 of the CSFV E2 protein (*7, 19*). The 10 different 15-mer sequences derived from the random synthetic peptide library were given at the lower part of the figure, aligning the YYEP core consensus region. Identical amino acid residues to the top sequence are indicated by dots. (B) Comparison of pestivirus E2 sequences around the YYEP region. The amino acid sequences were obtained from GenBank database (as given by their accession numbers): Weybridge, X71780; Brescia, M31768; C, Z46758; GPE, D49533; LPC, U35740; ALD, D49532; Alfort, J044358; Singer, L12455; SD-1, M96751; Osloss, M96687; C24V, L07496; BD78, U18330. The NADL sequence was taken from (*5*). Amino acid residues identical to the top Weybridge sequence are indicated by dots.

(*26*). At the N-terminus of the peptide, a biotin tag and the 4-residue spacer (Ser-Gly-Ser-Gly) were incorporated while the C-terminus of the peptide was kept free. After synthesis, the peptides were cleaved into a physiologically compatible buffer (0.166 *M* phosphate, pH 8.0) and tested for their capacity to bind mAb 4-9D4 in both indirect ELISA (I-ELISA) and competition ELISA (C-ELISA). In the I-ELISA, the peptides (diluted 1:100 in PBST from a 1 mg/ml stock) were immobilized via the biotin tag onto a streptavidin-coated plate by incubation at 37° for 1 hr. The plate was then incubated with mAb 4-9D4 (1:50) at 37° for 1 hr and bound antibody detected by a 0.5 hr incubation with horse radish peroxidase-conjugated sheep anti-mouse antibodies (1:5000). The results shown in Fig. 3A clearly demonstrated that the five peptides (numbers 2–6), which had significant binding to mAb 4-9D4, all shared the common sequence of YYEP. The C-ELISA analysis was carried out essentially as described in (*27*). Briefly, mAb 4-9D4 (1:100) was first incubated with individual peptides at 37° for 2 hr, spun for 10 min to remove any precipitated antigen/antibody complexes, then added to antigen-coated plates and incubated at 37° for 1 hr. Bound antibodies were detected as in I-ELISA. Again, only peptides 2–6 demonstrated strong competition (Fig. 3B). These results not only indicated that the core tetra-peptide YYEP constitutes the discrete antibody binding site from mAb 4-9D4, but also demonstrated that mAb 4-9D4 could bind to YYEP-containing peptides either displayed on phage surface or in free solution. It is interesting to note that among the five positive peptides number 3 had the weakest binding in both I- and C-ELISA analyses. This might be due to its high content of positively charged residues (KNKYYEPR, 3+, 1−), since the two flanking peptides carried less positive charge (peptide 2: 2+, 1−; peptide 4: 2+, 2−) and bind the mAb much better than peptide 3.

In summary, our data demonstrated that efficient and high resolution epitope mapping can be achieved using phage display technology. The complementary use of both gene-targeted epitope library and synthetic peptide

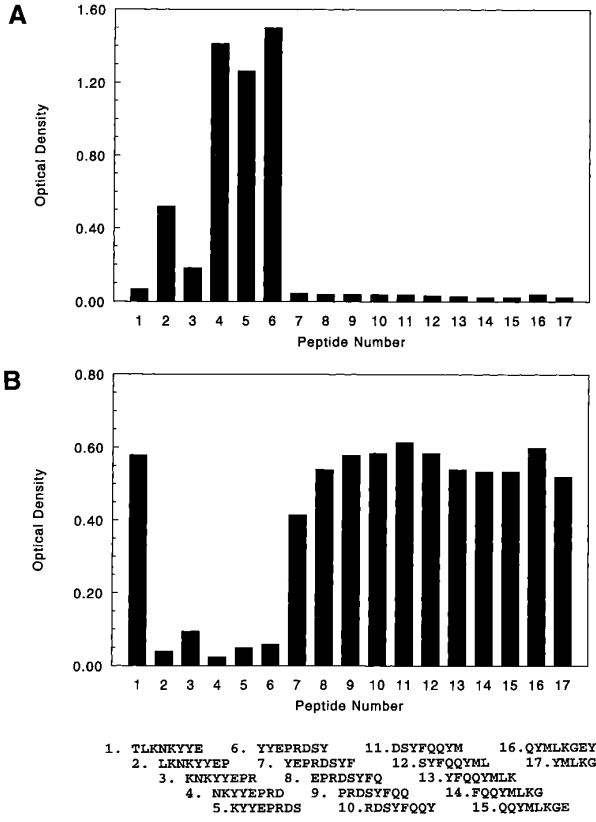

FIG. 3. Peptide ELISA analysis. Same peptides were used for both I-ELISA (A) and C-ELISA (B) analysis with the sequence of each peptide given at the bottom of B. Purified recombinant phage f1CSFV-D4 was used as ELISA antigen in the C-ELISA analysis. The readings given were the average of two separate determinations.

library further enhanced the usefulness of this new and powerful technology in epitope identification and characterization. The use of synthetic peptides allowed us to demonstrate the specific binding of mAb 4-9D4 to the core YYEP region within a totally different scaffold, i.e., peptides displayed on phage surface versus free peptides in solution.

To our knowledge, this is the first detailed epitope mapping of a CSFV structural protein although several antigenic domains, with a resolution in the range of 47 to 110 amino acid residues, have been defined previously (*17*). The identification of a novel linear epitope at the C-terminus of pestivirus E2 proteins will complement the use of other N-terminus-specific mAbs in further studies on the function and structure of this important envelope protein. In a recent proposed structural model of CSFV E2 protein (*18*), the C-terminal hydrophobic region (around 35–40 amino acid residues) is predicted to be the membrane-spanning anchor which is mostly buried in the membrane. According to this model, the YYEP-region (around 65 amino acid residues from the C-terminus of the E2 molecule) is located very close to the viral envelope in a highly folded structure. The results obtained from this study supports this model, indicating that the YYEP epitope is not accessible on virus surface nor in a tightly folded native E2 molecule. It will be interesting to see whether this part of the E2 molecule can be exposed by limited treatment of virus particles with various proteases.

The universal binding of mAb 4-9D4 toward all pestivirus E2 proteins in Western blot also provides a highly specific and conclusive genus-specific diagnosis tool for virus identification and characterization. Although genus-specific mAbs have been reported before, e.g., p80-specific mAbs generated against BVDV virus (*28*), to our knowledge 4-9D4 represents the first genus-specific mAb which reacts with a structural protein in Western blot and recognizes a well-defined and highly conserved epitope.

Finally, our preliminary data indicated that fusion of the YYEP sequence with an unrelated recombinant protein rendered the fusion protein reactive with mAb 4-9D4 (L.-F. Wang, unpublished results), pointing out the possibility of using YYEP as a small affinity tag for detection and purification of recombinant proteins, similar to several other epitope tags derived from viral proteins, including the 9-aa HA1-tag (*29*) and the 6-aa BTag, which has recently been developed by our group (*30*).

ACKNOWLEDGMENTS

We thank Professor G. P. Smith of University of Missouri, Columbia, for providing the f88-4 phage display random 15-mer peptide library, and G. Beddome and G. Crameri for technical assistance.

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